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Exploration and classification of chromatographic fingerprints as additional tool for identification and quality control of several *Artemisia* species

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ABSTRACT

The World Health Organization accepts chromatographic fingerprints as a tool for identification and quality control of herbal medicines. This is the first study in which the distinction, identification and quality control of four different *Artemisia* species, *i.e. Artemisia vulgaris*, *A. absinthium*, *A. annua* and *A. capillaris* samples, is performed based on the evaluation of entire chromatographic fingerprint profiles developed with identical experimental conditions. High-Performance Liquid Chromatography (HPLC) with Diode Array Detection (DAD) was used to develop the fingerprints. Application of factorial designs leads to methanol/water (80:20 (v/v)) as the best extraction solvent for the pulverised plant material and to a shaking bath for 30 min as extraction method. Further, so-called screening, optimisation and fine-tuning phases were performed during fingerprint development. Most information about the different *Artemisia* species, *i.e.* the highest number of separated peaks in the fingerprint, was acquired on four coupled Chromolith columns (100 mm × 4.6 mm I.D.). Trifluoroacetic acid 0.05% (v/v) was used as mobile-phase additive in a stepwise linear methanol/water gradient, *i.e.* 5, 34, 41, 72 and 95% (v/v) methanol at 0, 9, 30, 44 and 51 min, where the last mobile phase composition was kept isocratic till 60 min. One detection wavelength was selected to perform data analysis. The lowest similarity between the fingerprints of the four species was present at 214 nm. The HPLC/DAD method was applied on 199 herbal samples of the four *Artemisia* species, resulting in 357 fingerprints. The within- and between-day variation of the entire method, as well as the quality control fingerprints obtained during routine analysis, were found acceptable. The distinction of these *Artemisia* species was evaluated based on the entire chromatographic profiles, developed by a shared method, and visualised in score plots by means of the Principal Component Analysis (PCA) exploratory data-analysis technique. Samples of different quality could be indicated on the score plots. No multi-component analysis was required to reach the goal. Furthermore, differences related to the origin of some of the not-certified samples were shown. The importance of the specific herbal part used for its identification was also presented. In addition, no differences were observed among fingerprints of lyophilised or conditioned-air dried samples. Finally, a classification technique, Soft Independent Modelling by Class Analogy (SIMCA), was successfully evaluated as identification technique for unknown samples. Six additional *Artemisia* species (29 herbal samples) were identified as not belonging to any of the four modelled classes. The developed chromatographic fingerprints and the evaluation of the entire profiles provide an added value to the distinction, identification and quality control of the simultaneously investigated *Artemisia* species.

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1. Introduction

Proper recognition is of crucial importance in the quality control of herbal medicines or nutraceuticals. It helps avoiding problematic consequences of (coincidental) exchanges with other herbs. Not only different herbal species [1,2], but also differences in climate [3], growing region [4,5] or stage [6,7], harvest conditions [8], preservation and extract preparation [9] result in different contents in the plant material. Fingerprint analysis is accepted by regulatory authorities as a tool to identify herbal formulations and to assess their quality [10]. A fingerprint is a characteristic profile or pattern, which chemically represents the sample. In fingerprint analysis, the entire chromatogram is analysed. For multi-component analysis on the other hand, where detected and integrated peaks of chromatograms are evaluated, the quality of the data relies on the parameters used for peak detection and integration. A difference in sample quality is not only found in the main compounds but also in the low-concentration compounds. The latter are also taken better into account during fingerprint analysis. Moreover, since the information of all peaks is used in fingerprint analysis, it solves the problem of overlapping peaks. Therefore, fingerprint analysis might be an interesting way for identification and quality control of herbs, containing a large number of low amounts of unknown compounds. Once developed, the fingerprints need to be subjected to a proper data analysis, which depends on their purpose (*e.g.* identification or multivariate calibrations such as modelling pharmaceutical activities, *etc.*). An overview of theoretical aspects and examples can be found in [10]. To investigate if chromatographic fingerprint analysis can be a tool that allows a better identification and quality control for related species of a same herbal family, in this paper, chromatographic fingerprints are developed to distinguish and to control the quality of *Artemisia vulgaris* L., *A. absinthium* L., *A. annua* L. and *A. capillaris* Thunb, based on their entire profiles.

Artemisia species are widely used as traditional medicines or for their aromatic properties, due to a variety of compounds [1,2,7,11]. *A. vulgaris* and *A. absinthium* are both uterine stimulants [12]. The latter is traditionally also used to treat temporary loss of appetite and mild dyspeptic and gastrointestinal disorders [13,14]. *A. annua* (Sweet wormwood, Qinghao) is known for its antimalarial, antibacterial, anti-inflammatory and antitumoural activities [5,15,16]. According to the Chinese Pharmacopoeia, *A. scoparia* (Virgate Wormwood, Yinchen) may consist of *A. scoparia* Waldst. et Kit. (Bin Hao) or *A. capillaris* Thunb. (Yinchen Hao) [17]. *A. scoparia* is used as an anti-inflammatory, choleritic and diuretic agent for the treatment of hepatitis [4,8]. Recently also its antiproliferative potential was investigated [18]. The Belgian Law (KB 29 August 1997 [19]) lists some *Artemisia* species, like *A. annua* and *A. absinthium*, as dangerous herbs which are not allowed in the food industry unless the safety of the herb is proven (*i.e.* the concentrations of toxic compounds are below given limits) [19]. These restrictions are also found in the classification of the American Herbal Products Association [12]. *A. vulgaris* and *A. dranunculus* are listed as herbs that can be used in the food industry if a notification file is made and if the herb is divided in aliquots [19]. Such a notification file contains the type of product, a qualitative and quantitative list of ingredients, data about the therapeutic and toxic compounds, stability, *etc.*

Although the genus *Artemisia* contains an enormous number of species, most literature is found for *A. annua* [1,5–7,15–17,20–23] and *A. scoparia* [4,7,8,17,24,25]. Research on *A. vulgaris* [1,21,23,26], *A. absinthium* [13,14,21,23,26–29] or other species [2,21] was occasionally performed. Mainly the chemical composition is investigated and/or quantified or the therapeutic activity is evaluated in the literature. A summary is provided in Table 1. To our knowledge, only a few regulatory instances provide specific monographs for *Artemisia* species. Good Agricultural and Collection Practices (GACP) rules of the World Health Organization (WHO) are given for *A. annua* [20]. Nevertheless, the identification using normal-phase Thin Layer Chromatography (TLC) only considers one compound, *i.e.* artemisinin. The Chinese Pharmacopoeia contains monographs for *A. annua* and *A. scoparia* [17]. For *A. annua* the required TLC identification is comparable to that of the WHO, while for *A. scoparia* two different TLC methods are required, which consist of the identification of chlorogenic acid and scoparone, respectively. Both compounds are also assayed by two different HPLC methods [17]. The European Medicines Agency (EMA) provides a monograph and an assessment report on *A. absinthium* containing information on its use and some acceptance levels of certain (toxic) compounds, *e.g.* thujone. However, a quality control of the whole plant is hardly ever described [13,27]. The European Pharmacopoeia has a monograph for *A. absinthium* [28]. A normal-phase TLC identification is performed to detect artabsin and absinthin. In the 'Homöopathisches Arzneibuch' also normal-phase TLC is used to identify *A. vulgaris* and *A. absinthium*. For both herbs, different mobile phases and detection reagents are used [26]. For *A. vulgaris* the same normal-phase TLC method is used in the 'Hagers Handbuch der Pharmazeutischen Praxis' [21]. For *A. absinthium* two normal-phase TLC identification methods are given to detect different reference compounds. For *A. annua* and *A. abrotanum* other compounds, *i.e.* sesquiterpens and scopoletine, respectively, are identified [21]. Individual *Artemisia* species were in the past often a subject of study. A selection of the variety of chromatographic techniques used is overviewed. The (HP-)TLC technique is for instance used to detect artemisinin after derivatisation in *A. annua* [15]. Further, Gas Chromatography (GC) can be used to analyse volatile compounds [4,6]. In *A. capillaris*, 51 common compounds were found in four samples from different harvest locations [4]. Another paper investigated the metabolite variation in five different growth stages of *A. annua* by means of Partial Least Squares Discriminant Analysis (PLS-DA) of the metabolic fingerprints [6]. Sheu et al. [24] compared Micellar Electro Kinetic Capillary Chromatography (MEKCC) with Capillary Zone Electrophoresis (CZE) for the separation of 12 compounds in *A. capillaris*. In most non-regulatory studies, to reveal the chemical composition of *Artemisia* species, reversed-phase HPLC is used coupled with Ultra-Violet (UV) detection [30], Diode Array Detection [1,2,5,8,14,22,23,25], Evaporative Light Scattering Detection (ELSD) [16] or Mass Spectrometry (MS) [5,14,22,23]. Methods are developed to assay one substance, *e.g.* artemisinin [16,30], or several compounds [1,2,5,8,14,22,23,25]. A particle-based C_{18} column and isocratic elution is most often used [2,14–16]. Occasionally gradient elution is performed [2,22,23]. Different detection wavelengths are chosen in these analyses. For example, 280 nm is often used to detect flavonoids [5,22]. Wang et al. [25] used 254 nm to detect simultaneously different compounds in a Traditional Chinese Medicine containing *A. capillaris*. Tan et al. [8] simultaneously analysed thirteen compounds in *A. scoparia* at 280, 325, 345 and 355 nm [8]. Aberham et al. analysed sesquiterpene lactons and flavonoids simultaneously at 205 nm [14]. Artemisinin, an active compound of *A. annua*, does not have UV-absorbing properties and is thus often derivatisated to be detected, *e.g.* at 260 nm [30]. Alternatively, it is detected at short wavelengths [5]. Also ELSD or MS was used to detect non-UV-absorbing compounds [5,16,22].

From the above literature search, no shared method for the different species is available. Comparative analytical research between different *Artemisia* species, to our knowledge, has barely been published and is always based on the comparison of certain compounds. For example, Nikolova et al. [1] presented a Reversed Phase (RP)-HPLC/UV method to analyse external flavonoid aglucons from *A. annua* and *A. vulgaris*. A higher concentration of quercetagetin 3,6,7-trimethyl ether for *A. annua* and a higher